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Methoxychlor Affects the Decidual Cell Response of the Uterus but Not Other Progestational Parameters in Female Rats (Journal Version)

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16. ABSTRACT

The pesticide methoxychlor (MXC) is a proestrogen which is metabolized to a compound that has been shown to exhibit estrogenic activity in vivo and in vitro. Following long-term exposure of female rats to MXC, fertility is reduced and fetotoxicity is evident. However, the effects of MXC on several aspects of maternal reproductive physiology including the decidual cell response (DCR), ovarian weight, serum progesterone levels, and corpora lutea maintenance, have not been previously described. In the present study, the ability of MXC to interfere with progestational events essential for implantation and the maintenance of pregnancy in the rat was investigated. MXC was administered to pseudopregnant rats, decidualization was induced on day 4, and the DCR and related parameters were evaluated following sacrifice on day 9. DCR induction during estrone administration served as a positive control. MXC inhibited decidualization in a dose-dependent manner: while 100mg/kg/day had no effect, doses of 200, 300, 400, and 500mg/kg/day produced incremental inhibition of the response. No effects of MXC on ovarian weight, serum progesterone levels, or number of corpora lutea were observed. Estrone also inhibited the DCR in a dose dependent manner. The data suggest that the anti-fertility effect of MXC is mediated by a suppression of decidualization and that such suppression, which is characteristic of estrogenic compounds, is a direct uterine effect. In the DCR model system, the estrogenic activity of MXC is 1/20,000 times the activity of estrone.

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Methoxychlor Affects the Decidual Cell Response of the Uterus But Not Other Progestational Parameters in Female Rats[1,2]

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Methoxychlor Affects the Decidual Cell Response of the Uterus but not Other Progestational Parameters in Female Rats. CUMMINGS, A. M., AND GRAY, L. E., JR. (1987). *Toxicol. Appl. Pharmacol.* **90**, 330–336. The pesticide methoxychlor (MXC) is a proestrogen which is metabolized to a compound that has been shown to exhibit estrogenic activity *in vivo* and *in vitro*. Following long-term exposure of female rats to MXC, fertility is reduced and fetotoxicity is evident. However, the effects of MXC on several aspects of maternal reproductive physiology, including the decidual cell response (DCR), ovarian weight, serum progesterone levels, and corpora lutea maintenance, have not been previously described. In the present study, the ability of MXC to interfere with progestational events essential for implantation and the maintenance of pregnancy in the rat was investigated. MXC was administered to pseudopregnant rats, decidualization was induced on Day 4, and the DCR and related parameters were evaluated following euthanasia on Day 9. DCR induction during estrone administration served as a positive control. MXC inhibited decidualization in a dose-dependent manner: while 100 mg/kg/day had no effect, doses of 200, 300, 400, and 500 mg/kg/day produced incremental inhibition of the response. No effects of MXC on ovarian weight, serum progesterone levels, or number of corpora lutea were observed. Estrone also inhibited the DCR in a dose-dependent manner. The data suggest that the anti-fertility effect of MXC is mediated by a suppression of decidualization and that such suppression, which is characteristic of estrogenic compounds, is a direct uterine effect. In the DCR model system, the estrogenic activity of MXC is 1/20,000 times the activity of estrone.

Methoxychlor (MXC), a chlorinated hydrocarbon, is a pesticide that is much less acutely toxic than the closely related DDT and has an oral LD50 > 6000 mg/kg in the rat (Kapoor, 1970). Technical grade and purified MXC both show estrogenic activity *in vivo*. For example, purified MXC produces an increase in uterine wet weight 6 hr after administration to immature rats (Welch *et al.*, 1969). MXC is considered a proestrogen and is metabolized via *O*-demethylation by rat hepatic microsomes to an estrogenic compound which, *in vitro*, inhibits estrogen binding to the uterine cytosol estrogen receptor (Bulger *et al.*, 1985).

Previous research in our laboratories indicated deleterious effects of MXC on fertility in rats following subchronic exposure to the compound (Gray *et al.*, 1986). These findings agree with those seen by others during shorter-term administration of MXC in which fetotoxicity and teratogenicity were identified (Khera *et al.*, 1978; Harris *et al.*, 1974). The present study was designed to in-

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vestigate the effect of MXC on the capability of the uterus to undergo decidualization and thereby identify a strictly maternal effect independent from the compound's effects on the fetal compartment.

The decidual cell response (DCR) has long been used as a model for events associated with the implantation of blastocysts in receptive uteri (De Feo, 1967). It mimics the response of the uterus which occurs following implantation of blastocysts during early pregnancy. The DCR can be induced with appropriate artificial stimuli in animals previously stimulated to become pseudopregnant, a physiological state endocrinologically similar to early pregnancy. The response is gauged by the ability of the uterus (a) to respond maximally to a deciduogenic stimulus and (b) to undergo a period of rapid, organized growth resulting in a deciduoma. Uterine decidual tissue induced in the pseudopregnant rat is histologically and biochemically similar to the decidual tissue seen during early pregnancy, and both conditions are dependent upon the appropriate sequence and levels of estrogen, progesterone, and prolactin (De Feo, 1967). The successful DCR is indicated by a massive increase in uterine weight which differs both quantitatively and qualitatively from the increase in uterine weight seen following the administration of estrogen alone.

The DCR has been used to examine the effects on uterine responsivity of estrogen and progesterone (Velardo and Hisaw, 1951; Rothchild *et al.*, 1940), nicotine (Card and Mitchell, 1978), serotonin (Mitchell *et al.*, 1983), zineb (Sing and Spencer, 1981), rotenone (Spencer and Sing, 1982), and nicotinamide (Yochim, 1984). The morphological and endocrinological similarities between the DCR and early pregnancy make the DCR a simple and valuable technique for assessing not only endocrine status and uterine sensitivity for implantation but also for distinguishing fetotoxic and teratogenic effects from effects of xenobiotics on maternal physiology. In the current study, the DCR was used successfully to demonstrate a methoxychlor-induced inhibition of uterine decidualization and, thus, a direct maternal effect of the pesticide.

METHODS

General. Adult female Holtzman rats, obtained from Small Animal Supply Co. (SASCO) and weighing 190 to 265 g, were used. Animals were housed in pairs in clear plastic cages (20 × 25 × 47 cm) with heat-treated laboratory-grade pine shavings (Northeastern Products Corp., Warrensburg, NY) as litter. Purina rodent chow (5001) and water were given *ad libitum*. A photoperiod of 14 hr of light and 10 hr of darkness was maintained, with the midpoint of the light phase set at 1200 hr. The temperature in the animal room was 20–24°C with a relative humidity of 40–50%.

DCR. Estrous cycles of all animals were monitored throughout all experiments by daily vaginal smears, and only those rats exhibiting at least two consecutive 4- or 5-day cycles were used. Pseudopregnancy was induced by stimulation of the uterine cervix with a small brass rod on proestrus and estrus (De Feo, 1963). This procedure is a substitute for the stimulus, normally provided by the male rat during coitus, which induces the altered pattern of prolactin secretion required for the maintenance of corpora lutea and progesterone secretion. Day 0 of pseudopregnancy was defined as the last day of vaginal cornification, provided that significant leukocytic infiltration followed on Day 1. Deciduomata were induced surgically in all animals, by the knife-scratch uterine traumatization technique (De Feo, 1963), on Day 4 of pseudopregnancy between 1000 and 1200 hr. Uterine trauma at this time mimics the effect of an implanting blastocyst. In each rat, only the right uterine cornu was traumatized, and the left cornu remained untreated for comparison.

Block 1. MXC[3] was dissolved in and diluted with sesame oil. The compound was administered by oral gavage to pseudopregnant rats from Days 1 through 8. Each group of rats received MXC at doses of 0 (vehicle), 100, 200, or 400 mg/kg/day using dilutions of MXC to provide equal volumes of solution per kilogram body weight. These doses were determined from preliminary experiments which initially used doses based on literature values (Gray *et al.*, 1986). Animals were weighed and killed by cervical dislocation on Day 9 of pseudopregnancy. The uteri were removed, trimmed of fat, mesentery, and ovaries, and split at the cervical junction. Individual cornu were weighed to the nearest 0.10 mg. Uteri in which deciduomata were successfully induced appeared sausage-shaped and well vascularized. The change in the

[3] Laboratory-grade methoxychlor (MXC, approximately 98%; lot No. 62F-5054), estrone (lot No. 32F-0258), and sesame oil (lot No. 104F-0903) were purchased from Sigma Chemical Co.

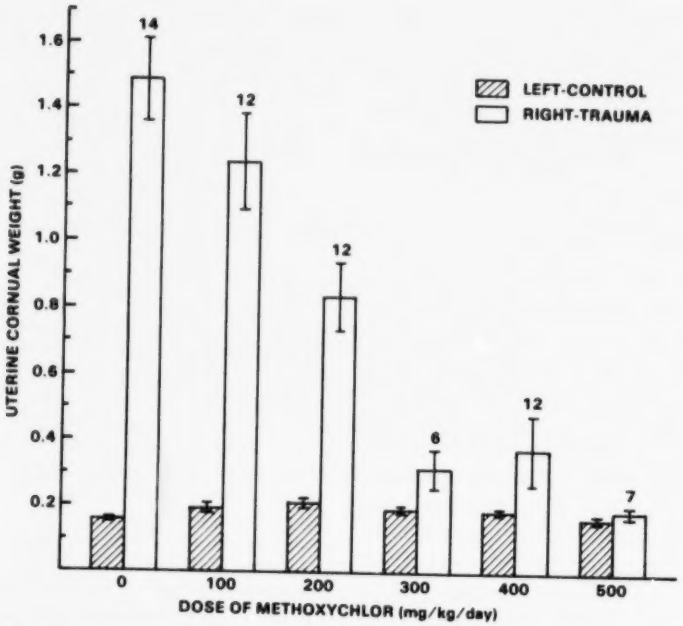

FIG. 1. Effect of methoxychlor on the decidual cell response (DCR). Groups of adult female rats were administered methoxychlor at the indicated doses during Days 1–8 of pseudopregnancy. Decidualization was induced by uterine trauma of the right uterine cornu of each rat on Day 4. Both left and right cornual weights were determined following euthanasia on Day 9. The values represent the means ± standard errors with the number of rats per group indicated above the error bars. Methoxychlor inhibited the DCR at 200, 300, 400, and 500 mg/kg/day ($p < 0.001$) but not at 100 mg/kg/day.

size of the decidua-bearing uteri is due to a huge increase in solid tissue mass resulting from the proliferation of differentiated decidual tissue; this growth differs radically from the uterine growth induced by estrogen alone.

Block 2. In the second block, groups of rats received 0, 100, 200, 300, 400, or 500 mg/kg/day of MXC from Days 1 through 8 of pseudopregnancy. Trunk blood was collected following weighing and decapitation on Day 9, and uteri were analyzed as in block 1. Serum was separated using serum separation tubes and centrifugation, and serum progesterone was measured by radioimmunoassay (RIA; Diagnostic Products). Ovaries were removed from the bursa, trimmed of fat and oviductal tissue, weighed, and preserved in buffered formalin. At a later time, ovaries were observed under a dissecting microscope and corpora lutea were counted.

Positive control. Estrone was dissolved in sesame oil and administered by sc injection from Day 1 through Day 8 of pseudopregnancy. Each group of rats received 0 (vehicle), 0.05, 0.25, 0.5, 1.0, 2.5, 5.0, or 10.0 μg estrone/rat/day in a volume of 0.10 ml. Animals were killed on Day 9, and uteri were removed and weighed as described.

Statistics. Cornual and body weights from rats in blocks 1 and 2 were pooled for analysis. Data for all parameters were analyzed for treatment and block effects by the general linear models (GLM) procedure after logarithmic transformation to correct for heterogeneity of variance (Helwig and Council, 1979). When significant effects on the overall ANOVA were detected, post hoc comparisons between treatments were made with *t* tests. Pearson correlation coefficients were also calculated.

RESULTS

Compared with the administration of vehicle alone (0), methoxychlor had no effect on the left (control) or right (DCR) cornual weight at a dose of 100 mg/kg/day (Fig. 1). However, at a dose of 200 mg/kg/day a partial inhibition of the DCR (right cornua) was observed ($p < 0.001$). Larger doses of MXC (300, 400, and 500 mg/kg/day) produced fur-

TABLE 1

BODY WEIGHT GAIN DURING METHOXYCHLOR ADMINISTRATION[a]

Dose MXC (mg/kg/day)	N	Body weight[b] Day 1 (g)	Body weight[b] Day 9 (g)	Body weight[c] change (g)
0	14	231.00 ± 4.23	240.43 ± 3.91	9.43 ± 2.33
100	12	221.50 ± 4.70	225.00 ± 3.95	3.50 ± 2.95
200	12	223.08 ± 4.83	225.00 ± 4.62	1.92 ± 2.38
300	6	232.17 ± 2.54	235.33 ± 6.76	3.16 ± 4.44
400	12	224.83 ± 5.63	226.67 ± 5.53	1.84 ± 2.97
500	7	240.14 ± 6.45	239.86 ± 10.20	−0.28 ± 4.80

[a] Methoxychlor was administered to groups of rats during Days 1–8 of pseudopregnancy at the indicated dosages. The DCR was induced on Day 4 and body weight was measured after euthanasia on Day 9. N = number of rats per treatment group.

[b] Data are expressed as the mean ± the standard error of the mean of the individual rat body weights on each day for each treatment.

[c] Data are expressed as the mean ± the standard error of the calculated difference in body weight of each rat in each group.

ther suppression of the decidual cell response until decidualization was totally inhibited by the administration of 500 mg/kg/day of the compound ($p < 0.001$). No significant increase in cornual weight was seen in the left, nontraumatized cornu from rats which received any dose of MXC. Analysis of the total body weight gain during the 8-day period of treatment with MXC showed no effect of the pesticide on this parameter (Table 1).

Methoxychlor did not affect any of the measured parameters associated with progestational physiology (Table 2). Ovarian weight, serum progesterone levels, and corpora lutea number showed no significant differences among treatment groups when analyzed by the GLM. Further, no correlation was found between the decidual cell response (right cornual weight) and any measured parameter for any treatment group.

The well-known inhibitory effect of estrogen on the DCR (Velardo and Hisaw, 1951; Yochim and De Feo, 1962, 1963) was demonstrated during the administration of estrone (Table 3). Where low levels of estrone had no effect on (0.05 and 0.25 μg/rat/day) or slightly augmented (0.5 μg/rat/day) the DCR, higher levels of the hormone suppressed the response either partially (1.0 and 2.5 μg/rat/day) or completely (5.0 and 10.0 μg/rat/day).

DISCUSSION

The normal reaction to decidual induction in the rat is a 10-fold increase in the weight of the traumatized cornu, while the weight of the unstimulated (control) cornu does not change. The traumatized cornu normally undergoes a decidualization process similar to that seen during early pregnancy; subepithelial stromal cells of the endometrium differentiate into decidual cells, and a rapid increase in cell size and number occurs (De Feo, 1967). The ability of the uterus to so respond depends, first, on a proper hormonal milieu which permits the development of sensitivity to decidualization on Day 4 of pregnancy or pseudopregnancy; the sensitivity is limited in time and is not obtainable except under strictly defined control by estrogen and progesterone (Yochim and De Feo, 1963). Second, the growth of the decidual reaction after Day 4 will proceed only if the uterine environment required for differentiation and proliferation is maintained, mainly via adequate support by estrogen and progesterone (Yochim and De Feo, 1962). The presence of excess es-

TABLE 2

EFFECTS OF METHOXYCHLOR ON PROGESTATIONAL PARAMETERS[a]

Dose MXC (mg/kg/day)	N	Ovarian weight (mg/rat)	Serum progesterone (ng/ml)	Corpora lutea (No./rat)
0	7	69.81 ± 7.83	115.5 ± 15.57	15.43 ± 1.17
100	6	56.05 ± 2.97	75.03 ± 9.00	15.00 ± 0.82
200	6	57.62 ± 1.77	71.31 ± 11.26	14.67 ± 0.88
300	6	53.93 ± 4.57	61.85 ± 7.84	15.50 ± 1.09
400	6	55.52 ± 2.71	83.03 ± 11.84	14.50 ± 1.02
500	7	56.30 ± 4.67	115.7 ± 26.74	13.71 ± 0.61

[a] Methoxychlor was administered to groups of rats during Days 1–8 of pseudopregnancy at the indicated dosages. The DCR was induced on Day 4 and ovarian weight, serum progesterone concentration, and corpora lutea number were examined following euthanasia on Day 9. *N* = number of rats per treatment group. Data are expressed as the means ± the standard error of the mean.

trogen renders the uterus incapable of full decidualization, independent of ovarian function (Yochim and De Feo, 1962, 1963; Velardo and Hisaw, 1951). This is in contrast to the classic bioassay in which estrogen promotes an increase in uterine weight in immature or ovariectomized adult rats.

Methoxychlor requires metabolic activation *in vitro*, and the estrogenic activity of the pesticide is well documented (Bulger *et al.*, 1978, 1985; Bulger and Kupfer, 1983; Welch *et al.*, 1969; Kupfer and Bulger, 1979). In a manner similar to that seen during the administration of estrone, MXC had no effect on the DCR at low doses but produced increasing levels of inhibition of the DCR as the dose was increased. It should be noted that, in the present study, the administration of MXC was limited to the postovulatory, periimplantation period, and the inhibitory effect on decidualization was seen during this short-term administration.

TABLE 3

EFFECT OF ESTRONE ON THE DECIDUAL CELL RESPONSE[a]

Dose of estrone (μg/rat/day)	N	Left cornua[b] weight (g)	Right cornua[b] weight (g)	Body weight[b] change (g)
0	2	0.2453	1.6905	25
0.05	3	0.3852[c]	1.6000	19
0.25	2	0.1540	1.5681	12.5
0.50	2	0.2776	1.9619	26
1.00	2	0.2103	1.3044	12
2.50	2	0.2106	0.9268	18.5
5.00	2	0.1990	0.2217	15
10.00	2	0.2250	0.2506	18

[a] Estrone was administered by sc injection to groups of rats during Days 1–8 of pseudopregnancy and decidualization was induced on Day 4. Cornual and body weights were measured following euthanasia on Day 9. *N* = number of rats per group.

[b] Data are expressed as the mean of each group.

[c] Some left cornua were inadvertently traumatized near the cervical junction and showed a small, local response.

If the inhibition of decidualization by MXC is to be considered a mechanism of the suppression of fertility by the compound, then a comparison between the doses of MXC required to inhibit the DCR and the doses which produce embryotoxicity should be made. In a study by Khera *et al.* (1978), MXC was administered to rats by gastric intubation from Days 6 through 15 of gestation, a dosing duration which is comparable to that described here. The authors report that the incidence of malformations in the pups was not significant at 50 and 100 mg/kg/day MXC. However, MXC at 200 mg/kg/day decreased fetal weight and number of live fetuses per litter. Increased numbers of resorption sites and dead fetuses were found in dams given 200 or 400 mg/kg/day. Thus, MXC at 50 or 100 mg/kg/day, with short-term administration, is neither embryotoxic nor inhibitory to the DCR, whereas at 200 or 400 mg/kg/day the compound is embryotoxic and blocks the DCR (Khera *et al.*, 1978). These data support the concept that MXC suppresses fertility, at least in part, via an inhibition of uterine decidualization.

Two lines of evidence support the concept that the inhibition of decidualization by MXC is a direct uterine effect. The inhibitory action of estrogen on the DCR is independent of the presence of ovaries (Yochim and De Feo, 1962, 1963), and methoxychlor, in light of previous evidence in other systems, is likely to exert its activity in a manner similar to that of other estrogens. Second, the fact that MXC did not affect ovarian weight, serum progesterone levels, or corpora lutea number implies that ovarian function and corpus luteum maintainance were not compromised by MXC administration. Similarly, there was no correlation between the inhibition of the DCR and these measured progestational parameters during MXC administration. The measured progesterone levels reported here are similar to those measured previously (Pepe and Rothchild, 1974).

A comparison of the dose-related effects of estrone and MXC on the DCR permits an estimation of the relative estrogenicity of MXC under the conditions of these experiments. Inhibition of the DCR by methoxychlor (at 200 mg/kg/day) required a dose of that compound which was 20,000 times the necessary dose of estrone to produce a similar inhibition (2.5 μg estrone/day; approximately equivalent to 10 μg/kg/day). By contrast, the 18-hr glycogen assay using the immature rat uterus indicated that technical-grade MXC was 10,000 times less estrogenic than diethylstilbestrol (DES) (Bitman and Cecil, 1970). Ousterhout *et al.* (1981) showed that MXC was relatively inactive when assayed to measure the ability to inhibit [^{3}H]estradiol binding to the rat uterine receptor *in vitro* and that the *bis*-phenolic metabolite of MXC was 100 times less active than DES in the same assay. These and other reports demonstrate that a measure of a compound's estrogenicity depends largely on the conditions of the assay. The DCR technique is not only useful as an assay for estrogenicity but is also an important method for evaluating uterine integrity and reproductive physiology under conditions of toxic insult.

The significance of the data reported here lies in the fact that (1) uterine decidualization is required for normal implantation, placentation, and, therefore, normal fecundity in rats and (2) the DCR method separates potential effects of administered compounds on the fertilized egg from effects on maternal reproductive physiology including uterine function. The suppression of fertility observed during MXC administration may be mediated by any of a variety of mechanisms including the hypothalamic–pituitary–gonadal axis or fetotoxicity. The data reported here show that at least one mechanism operating during MXC's suppression of fertility is an inhibition of the uterine capability for decidualization; this mechanism may be related to the pesticide's estrogenic activity. Such an inhibition of decidualization may involve either a blockade of the development of uterine sensitivity or a suppression of the growth of decidual tissue; alternatively, both mechanisms may be active.

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